CONTROL SYSTEM AND APPARATUS FOR
USE WITH LASER EXCITATION OR IONIZATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   This application is a continuation of PCT/US02/02548, filed January 28, 2002, which claims priority to U.S. Provisional Application Serial No. 60/265,133, filed January 30, 2001.

5   ## BACKGROUND AND SUMMARY OF THE INVENTION

[0002]   The present invention generally relates to control systems and apparatuses for use with laser excitation or ionization, and more particularly to a system and apparatus which employs a laser, pulse shaper, mass spectrometer and electrical control system.

10   [0003]   Conventionally, laser desorption mass spectrometry has been used with a fixed laser beam pulse shape and computers for simple chemical analysis processes on purified molecules with or without a matrix. The laser beam pulse shape was not considered an important parameter and was not modified; whatever fixed shape was set by the manufacturer for the ultraviolet laser was used in the

15   tests. The general concept of typically laser selective ion formation from molecules in a molecular beam is disclosed in the following publication: Assion et al., "Control of Chemical Reactions by Feedback-Optimized Phase-Shaped Femtosecond Laser

Pulses," *Science*, Vol. 282, page 919 (October 30, 1998). The pulse shaping

process with a learning algorithm is disclosed in Judson et al., "Teaching Lasers to

Control Molecules," *Physical Review Letters*, Vol. 68, No. 10, page 1500 (March 9,

1992). It is noteworthy, however, that the Assion article discloses use of an 80

5   femtosecond laser pulse and requires molecules to be isolated in a molecular beam,

while the Judson article discloses use of a one nanosecond laser pulse and is purely

conceptual as it does not include experimental results.

        [0004]   It is also known to employ nanosecond lasers for matrix-assisted

laser desorption ionization (hereinafter "MALDI"). Examples of this are disclosed in

10   U.S. Patent No. 6,130,426 entitled "Kinetic Energy Focusing for Pulsed Ion

Desorption Mass Spectrometry" which issued to Laukien et al. on October 10, 2000,

and U.S. Patent No. 6,111,251 entitled "Method and Apparatus for MALDI Analysis"

which issued to Hillenkamp on August 29, 2000; both of these patents are

incorporated by reference herein.

15   **[0005]**   Until recently, commercially practical femtosecond lasers have been

unavailable. For example, lasers which can generate 10 femtosecond or less laser

pulse durations have traditionally been extremely expensive, required unrealistically

high electrical energy consumption (for extensive cooling, by way of example) and

depended on laser dyes that had to be replenished every month thereby leading to

20   commercial impracticality. The efficiency of sub-10 femtosecond lasers was not

practical until the year 2000 because of the prior need for dyes and flash lamps

instead of YAG and Ti: Sapphire crystals pumped by light or laser emitting diodes.

[0006]   Furthermore, the traditional role of the laser in a mass spectrometer with MALDI is to provide energy to the matrix molecules, wherein this energy dissipates and causes evaporation and ionization of the protein analyte dissolved in it.   The laser, therefore, plays an indirect role that depends on energy transfer processes that may take from picoseconds to microseconds.   Because excitation is indirect, pulse wavelength has not been found to cause significant differences in the outcome.   Direct laser excitation of the proteins with nanosecond lasers typically causes the proteins to char.

[0007]   In contrast, the present invention uses a different approach to MALDI in which the laser plays a more active and direct role in the ionization and even selective fragmentation of the analyte proteins.   Shaped femtosecond pulses are required to achieve this goal.   The optimum pulse shape cannot be found using the traditional laser sources, and trial and error.   This is because the search for an optimal laser pulse shape involves a very wide range of possibilities.   For example, if a 100 femtosecond laser pulse is used to produce pulse trains as long as several picoseconds in duration, splitting the laser beam spectrum into at least 100 spectral components is required since the length of the pulse is roughly inversely proportional to the band width.   Since each component can be attenuated in 10 steps or phases shifted over 10 angles, then there are $(10 \ X10)^{100}$ different possible pulse shapes, and it would be impractical to systemically explore even a subset of these pulse shapes through conventional trial and error methods.

[0008]   Laser induced, selective chemical bond cleavage has also been explored but with fairly limited success. It is believed that very simple molecules, such a HOD (partially deuterated water), have had only the OH and OD bonds cleaved with a nanosecond narrow line laser to vibrationally excite the specimen and then an ultraviolet laser pulse was employed to perform the cleaving. The desired laser frequency for vibrational excitation could be determined a priori in the gas-phase sample. More importantly, the HOD molecule is unique because the energy can be deposited in one of the bonds and it remains there for very long times, which are longer than nanoseconds. For the HOD experiments using selective bond excitation, no appreciable pulse shaping was used. This method was not known to have been employed for a protein or MALDI process, and was not known to have been successfully used for any other atomic bonds in other molecules, especially not in a condensed phase. It is also noteworthy that MALDI, with a matrix, has been used in an attempt to perform limited bond cleavage, as is discussed in U.S. Patent No. 6,156,527 entitled "Characterizing Polypeptides" which issued to Schmidt et al. on December 5, 2000, and is incorporated by reference herein. However, the approach of Schmidt et al. does not modify and optimize the laser pulse shape or other laser properties to achieve limited bond cleavage.

[0009]   In accordance with the present invention, a control system and apparatus for use with laser excitation or ionization is provided. In another aspect of the present invention, the apparatus includes a laser, pulse shaper, detection device and control system. A further aspect of the present invention employs a femtosecond

laser and a mass spectrometer. In yet another aspect of the present invention, the control system and apparatus are used in a MALDI process. Still another aspect of the present invention employs the control system and apparatus to cleave chemical bonds in a specimen and/or to determine the amino acid sequence of a protein specimen. Photodynamic therapy and fiber optic communication systems use the laser excitation apparatus with additional aspects of the present invention. A method of ionizing and determining a characteristic of a specimen is also provided.

[0010] The control system and apparatus of the present invention are advantageous over conventional constructions since the present invention allows for analysis and identification of constituents of complex and unknown molecules, such as those used in a MALDI process or proteins, in a relatively quick and automated manner. The present invention advantageously determines optimum laser conditions for maximizing the sensitivity of MALDI based protein sequencing, and to examine ion formation efficiencies for various matrices using tailored laser pulses. The present invention is also advantageously used to control the degree and type of fragmentation for automated protein sequencing. Furthermore, the adaptive laser source permits the optimal desorption from an insoluble protein source and allows for ionization analysis of a protein with or without a matrix.

[0011] The present invention is advantageous by employing ultra-fast laser beam pulses which can be repeatedly transmitted onto a specimen at least 1,000 times without replacing the specimen and without significant degradation of results. The ultra-fast laser also does not over-heat or "cook" a specimen, such as a protein.

Recent improvements and efficiencies of femtosecond lasers have allowed for their commercially practical usefulness with the present invention. The automated feedback and pulse shaping of the control system of the present invention enhances signal-to-background sensitivity, especially for MALDI-based protein sequencing,

5   while also statistically optimizing the process; this leads to significant time, cost and accuracy improvements. Additional advantages and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

10   **[0012]**   Figures 1 through 3 are diagrammatic views showing a first preferred embodiment of a control system and apparatus of the present invention;

[0013]   Figure 4 is a flow chart showing the operation of the first preferred embodiment control system and apparatus;

[0014]   Figure 5A through 7B are sets of laser beam pulse shapes

15   employed with the first preferred embodiment control system and apparatus;

[0015]   Figure 8 is an exemplary ionization fragmentation chart employed with the first preferred embodiment control system and apparatus;

[0016]   Figures 9 through 10B are flow charts for the method and computer software operation employed with the first preferred embodiment control system and

20   apparatus;

[0017] Figure 11 is an exemplary molecular structure cleaved by the first preferred embodiment control system and apparatus;

[0018] Figure 12 is a diagrammatic view showing a second preferred embodiment of a laser excitation apparatus of the present invention;

[0019] Figures 13A-13C are schematic and graphical representations of two photon and three photon induced fluorescence employed with the laser excitation apparatus;

[0020] Figures 14A-14H are sets of laser beam pulse shapes employed with the laser excitation apparatus for two and three photon induced fluorescence;

[0021] Figures 15A-15G are sets of laser beam pulse shapes employed with the laser excitation apparatus for two and three photon induced fluorescence;

[0022] Figures 16A-16F are sets of pie charts and laser beam pulse shape graphs showing contrast ratios of the laser excitation apparatus;

[0023] Figure 17 is a diagrammatic view showing third and fourth preferred embodiments of the present invention laser excitation apparatus applied to optical coherence tomography and photodynamic therapy;

[0024] Figures 18A – 18C are graphs showing the laser beam pulse spectrum employed with the laser excitation apparatus;

[0025] Figures 19A and 19B are graphs showing the calculated two and three photon absorption probability employed with the laser excitation apparatus;

[0026] Figures 20A - 20C are graphs showing the calculated two and three photon absorption probability employed with the laser excitation apparatus; and

[0027] Figure 21 is a diagrammatic view showing a fifth preferred embodiment of the present invention laser excitation apparatus applied to fiber optic communications.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

5   [0028] The first preferred embodiment of a control system and apparatus 21 of the present invention for use with laser excitation or ionization is generally shown in Figures 1 and 2. Apparatus 21 includes a femtosecond laser 23, an upstream grating 25, an upstream convex mirror 27, a laser beam pulse shaper 29, a downstream concave mirror 31, a downstream grating 33, a matrix-assisted laser

10   desorption ionization device 35, and a personal computer 37. Personal computer 37 has a microprocessor based electrical control system, an output screen, a data storage device, an input keyboard, and a removable disk. More specifically, the MALDI device provides a time-of-flight mass spectrometer ("TOF MS") 39. A sample or specimen 41 to be analyzed is placed within mass spectrometer 39. Bursts or

15   pulses of a laser beam 43 are emitted from laser 23, through the optics 25, 27, 31 and 33, as well as through pulse shaper 29, and onto specimen 41; this causes fragmentation and ionization of a top layer of the specimen for detection and sensing by mass spectrometer 39 for further evaluation, analysis, comparison and subsequent control by personal computer 37.

20   [0029] The laser is preferably an ultra-fast femtosecond and high peak intensity (with a typical peak greater than 10^{10} watts/cm^2) laser which preferably

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emits laser beam pulses of less than 100 femtosecond duration, and more preferably at or less than 50 femtoseconds, and for certain applications (such as, but not limited to, sequencing) even more preferably at or less than 10 femtosecond duration, for each pulse burst or shot. The intense optical pulses that are required to modify

5   material are formed in a Kerr-Lens modelocked titanium sapphire oscillator. Such lasers are capable of producing hundreds of nanometers of coherent bandwidth, although only about 10 nm are typically used. The output is amplified in a 1 kHz regenerative chirped pulsed amplifier. The output pulse is typically 100 fs long with a central wavelength of out 790 nm and total pulse energy of 0.1 to 1 mJ. Preferred

10   lasers include: the Hurricane model from Spectra Physics Inc., which is diode pumped and gives 0.8 mJ per pulse with sub-50 fs pulses at 1 kHz; and the CPA-2001+ model from Clark-MXR Inc., which gives 1.3 mJ per pulse with sub-150 fs pulses at 1 kHz, pumping a Clark-MXR Inc. non-collinear parametric amplifier (hereinafter "NOPA") which produces 0.2 mJ per pulse, and is capable of generating

15   sub-20 fs pulses. This NOPA system can even produce pulses between 10 fs and 4.5 fs.

[0030]   A VESTEC 2000 MALDI TOF mass spectrometer is believed to be suitable for this invention, although most commercial MALDI instruments can be adapted with the femtosecond laser, pulse shaper and feedback learning control

20   method described herein. During extraction, all of the ions obtain the same energy in the 30-kV ion acceleration region, and because K.E. = ½ mv^2, the lightest ions achieve the highest velocity and, thus, reach the detector first. This transient (for

example, having a duration of 300 microseconds) mass spectrum is recorded by a transient recorder at the detector. It is common practice to sum many (10-100) of this transient mass spectra to produce a sound spectrum from an ion-counting statistics criterion.

[0031]   In Figure 3, a conceptual schematic is shown outlining the four probable mechanisms theorizing where the proton transfer takes place. The laser impinges on matrix crystal specimen 41 creating a plume of highly charged particles. Some of the particle are proton rich, such as $M2H^+$ and are good proton donors. Gas phase collisions with the large and slow moving proteins may lead to the formation of the protonated analyte. Other gas-borne particles include clusters containing the naturally charged analyte and matrix molecules. As the clusters evaporate, the charged analyte remains. Alternatively, the laser causes local melting of the crystal. The proton transfer occurs in the liquid phase between the excited state (high acidity) matrix molecules and the analyte. Finally, the proton transfer could also occur in the solid phase. The excitons created by the laser near the protein can relax by reactive processes such as proton transfer. At present, it is unknown where the ionization step takes place.

[0032]   A Fourier plane pulse shaper is preferably used with the present invention. Ultra-fast laser pulses contain from one to fifty optical cycles, and last only a few femtoseconds. This is much faster than most current electronics and therefore shaping with fast time gates is very difficult. On the other hand, as a consequence of the uncertainty principle, the optical spectrum spans tens to hundreds of nanometers.

Such a large bandwidth is relatively easy to measure and to filter, and there are several techniques to shape the spectrum in the frequency domain, and thereby shape the temporal pulse upon recompression.

[0033] In order to access the frequency domain and the individual frequency components that comprise the pulse, a geometric arrangement is employed, using two back-to-back spectrometers. The spectrometers are especially designed to introduce no net temporal dispersion: that is, all colors pass through the spectrometers within the same amount of time. The first spectrometer (including grating 25 and mirror 27) spreads the unshaped pulse spectrum along a line according to its dispersion function $y(\alpha)$. The light intercepts spatial amplitude and phase mask pulse shaper 29 at this point. The mask output then forms the entrance to a second spectrometer (including grating 33 and mirror 31) which recombines the colors into a single shaped pulse.

[0034] The heart of pulse shaper 29 is the programmable 256 pixel liquid-crystal mask (consisting of two overlapping 128 pixel liquid crystal arrays) that is placed at the Fourier plane. This mask must be capable of either attenuating the individual colors or shifting their phase. For alternate embodiment pulse shapers, two different electronically programmable masks that are capable of controlling both amplitude and phase have been demonstrated: a liquid crystal display ("LCD") and an acousto-optic modulator ("AOM"). A LCD pulse shaper can be obtained from CRI Co. and has a modulator electronic driver.

[0035] The AOM consists of an anti-reflection coated Tellurium Dioxide (TeO2) crystal with a piezo electric transducer glued onto one end. The central frequency of the acoustic wave is $\alpha c/2\pi$ = 200 MHz. The acoustic velocity vs in the crystal is 4.2 km/s and the light pulse spends less than 10ps in the crystal, so the acoustic wave moves less than 0.002 λ acoustic during the transit of the light field through the crystal. Since the acoustic wave is essentially frozen as the optical pulse travels through the crystal, the complex amplitude of the acoustic wave traveling through the crystal in the y direction, $A(t)\cos\alpha ct = A(y/vs)\cos\alpha ct$, is mapped onto the optical field $E(\alpha)$ as it passes through the AOM. If some of the dispersed optical field encounters a weak acoustic wave, that frequency is attenuated; if the acoustic wave carrier is shifted by phase angle $\varnothing$, that phase shift is imposed on the optical field. This pulse shaper has a total efficiency of about 20% including the diffraction efficiency of the AOM and the diffraction efficiency of the gratings. The diffracted light is used and the undiffracted "zero order" beam is blocked, to allow full modulation of both amplitude and phase in the shaped beam. The shaped beam than has the form

$$[1] \quad E_{shaped}(\omega) = E_{input}(\omega)x\alpha(\omega)xe^{i\phi(\omega)t}$$

where $a(\omega)e^{i\phi(\omega)} = A[y(\omega)/v_s]$; α is the frequency, and e is a constant.

[0036] The shaped pulses are measured using spectral interferometry. In this technique, the shaped laser pulse is joined with an unshaped reference pulse on a beam splitter, and then the combined pulses are analyzed in a spectrometer. The

signal corresponds to a spectrally resolved interference. If the reference pulse is known to have a flat spectral phase, then the amplitude beating of the output beam is a direct measure of the spectral phase function.

[0037] The pulse shaping devices and methods can be further achieved as discussed within the following publications: A.M. Weiner, "Femtosecond Pulse Shaping Using Spatial Light Modulators," *Review of Scientific Instruments*, Vol. 71, No. 5, P. 1929 (May 2000); and J.X. Tull; "High-Resolution, Ultrafast Laser Pulse Shaping and its Applications," *Advances in Magnetic and Optical Resonance*, Vol. 20, P. 1 (1997). Fixed pulse shaping optics, such as chirped mirrors, can also be employed as will be discussed further hereinafter.

[0038] The transform limited pulses ("TL"), having all their frequencies in phase, are fed into the pulse shaper where curved mirror 27 focuses the spectrum onto Fourier plane 29. Changes in the phase ø and amplitude A of the spectral components indicated by the computer are used to tailor the laser pulse before reconstruction with second curved mirror 31 and grating 33. Once compressed, the shaped pulse is directed to mass spectrometer 39 for evaluation. The Fourier transform relationship between the time and the frequency domain allows us to calculate the necessary mask to create a certain shaped pulse. These calculations are based on

[2] $$f(v) = \frac{1}{2\pi} \int_{-\infty}^{\infty} f(t)e^{i2\pi vct} dt$$

and

[3] $$f(t) = \int_0^0 f(v)e^{-i2\pi v ct}dv$$

where v is the frequency in wave numbers, t is the time, and c is the speed of light. As shown in Figures 5A and B an amplitude mask blocking a portion of the spectrum of a laser pulse at the Fourier plane leads to the formation of large periodic wings in

5   the shaped pulse. When the phase of different frequencies in the laser pulses changes in sign, as shown in Figures 6A and B, the shaped pulse becomes a pair of out of phase pulses. Combinations of phase and amplitude masks can be used to create complex shaped pulses. The ideal pulse sequence for laser-desorption may be the combination of a long pulse to melt the matrix and vaporize the analyte

10   combined with a fast pulse to cause multiphoton ionization. This sequence is based on the observation that below threshold laser excitation in MALDI generates a plume of material with very little ionization, and the fast pulse at the tail of the sequence would provide the prompt ionization. In Figures 7A and B, the formation of such a pulse is illustrated. Notice that the sharp femtosecond pulse is preceded by a long

15   picosecond pulse.

[0039] The phase and amplitude masks of the pulse shaper are controlled by computer. The adaptive laser source is part of a learning feedback method that modifies the laser pulse shape based on its success at optimizing the yield of charged proteins. Traditionally in MALDI, the laser plays a relatively passive role as

20   the energy source. In the present application, the laser pulse shape takes a more dynamic role. Pulse shapes are envisioned which include sequences of pulses

where each pulse in the sequence plays a different role, for example, melting, excitation, selective fragmentation, proton transfer and evaporation.

[0040] The physical process runs itself by means of an intelligent "feedback" method by means of an intelligent loop. The learning method tries various pulse shapes, assesses their success in achieving the desired target excitation, and uses the knowledge gained in this way to improve the pulse shapes on subsequent laser shots, all with only minimal intervention of the researcher or system user. Changing conditions are automatically corrected within the learning method or feedback loop.

[0041] Reference should now be made to Figure 4. The feedback software is implemented with an initial random population of pulse shapes. Each pulse shape is characterized by a series of numbers that specify the spectral phase and amplitude in each of the wavelengths or frequencies within the pulse. This particular parameterization for the pulse shape is itself subject to optimization; this makes the algorithm adaptive. The most important part of the method is the test for fitness of a given pulse shape. Each pulse shape is tested for its ability to generate the result that most resembles a target selected in advance. For example, the program will calculate the ratio between the amplitude at the desired protein signal and the background. Once relative success is quantified, a new generation of pulse shapes is produced by mating different parts of the amplitude and phase information from pairs of the most fit pulse shapes from the current generation. In addition, the method prescribes a small probability (5%) of random changes or mutations in the

successful pulse shapes. Furthermore, a new set (10%) of random pulses are introduced in order to explore entirely new regions in the parameter space. This basic series of processes is iterated until the fitness converges on a "best" value.

[0042] The convergence and robustness of the feedback method solutions

5 can be measured in two different ways. First, the variance in the amplitude and phase information itself can be monitored. As the feedback method converges on a solution, the values fall into a narrow range that produces the best result. Over the course of many generations, some of the parameters become very stable, which is an indication that those spectral phases and amplitudes are particularly important for

10 driving the process that determines fitness. Secondly, the information for different initial conditions is monitored. If the feedback method is working it should converge to a similar result.

[0043] New sets of parameters are created by modifying and combining elements of previous pulse shapes to create new ones. This is carried out by

15 statistical operators that act on the phases and amplitudes of the pulse shapes. Operators that can be used include multi-point crossover, mutation, averaging, creep, smoothing, choice of phase and amplitude basis, and polynomial phase mutation. Crossover exchanges one or more sections of the phase and amplitude parameters from each of two or more pulse shapes. The resulting pulse shapes are then tested.

20 Mutation randomly alters individual phases or amplitudes in a pulse shape. Averaging produces pulse shapes by averaging the values of two or more pulse shapes. Creep is mutation where the final parameter value is constrained to fall

close to the initial value. Smoothing averages nearby phase or amplitude values in the pulse shape. Polynomial-phase mutation produces pulse shapes by replacing a portion of the parameters with a polynomial fit.

[0044] A well-chosen set of operators can greatly enhance the performance of the feedback method and lend additional physical insight. However, the proper choice is usually far from obvious, so the method is allowed to adapt itself by letting it choose how often to use a given operator to produce new pulse shapes. The use of adaptive operators helps speed up convergence, and, perhaps more importantly, it helps shed light on the control mechanism at work. For example, crossover is more effective in the beginning of the algorithm when there is maximal uncertainty, since it does a good job of mixing up the information of the initial pulse shapes. It becomes less effective as the feedback method converges to the best solutions, since at this point there is much less change in the parameters, so there is no longer a need to drastically change the information. Ideally, the learning program learns from its past mistakes and does not test possible pulse shapes which it now knows will fail, which saves a considerable amount of computing time.

[0045] For each pulse shape, a number of spectra will be obtained. The number of laser shots that are averaged per pulse shape will depend on achievement of a statistically significant spectrum. At first, when the pulse shapes are the result of random phases and amplitudes we imagine that up to 1000 repetitions may be needed to distinguish the more efficient pulse shapes. This will allow one pulse shape per second. As the selection process proceeds large gains in efficiency can

be expected. The final stages of the optimization may be carried out at a rate of 100 different pulse shapes per second. The goal is to reach single pulse, femto-mol sensitivity.

[0046]   The learning feedback software employed in the present invention control system and apparatus is more fully described as follows. The preliminary investigation method and computer software steps for analyzing a pre-test unknown sample or specimen can be observed in Figure 9. For any new system, the test should start with pre-defined pulse shapes in order to obtain a basic understanding of the system. Among the pre-defined pulses, the shortest pulse is expected to ionize molecules on the surface of the sample with minimum decomposition, the longest pulse is expected to mimic the nanosecond experiments where the singly protonated protein may be observed. It is also noteworthy to vary the delay between two laser pulses from a few picoseconds to a few nanoseconds in order to appreciate the time scales involved. The manual inputs of steps A through C will be initially performed by the system operator or user through entering input data into the personal computer. The microprocessor within personal computer 37 will then control laser 23 in accordance with step D, receive an essentially real time feedback input signal from mass spectrometer 39 in accordance with step F and then perform calculations, comparisons and evaluations in accordance with steps G, H and I. These automated steps can be substituted with manual user calculations and decisions if desired based on personal computer outputs.

[0047]   The objective of the software routine of Figure 9 is to aid in the selection of sample targets for further testing iterations for subsequent criteria data input.   An optional alternate embodiment subroutine includes shooting long laser beam pulses then quick short laser beam pulses, with a separation set by an optical delay of less than ten nanoseconds.   The short pulse of approximately 50 femtoseconds is performed in order to look for fragmentation and the matrix mass. Laser beam pulses of between approximately ten and 100 picoseconds are performed to look for the parent mass.   The ultrafast laser beam pulse durations employed with the present invention advantageously allow for approximately 1000 laser beam shots at a single sample or specimen without significant degradation of the specimen; this allows for quicker and less expensive usage of the apparatus while also encouraging statistically more accurate results.   The long and short pulse combinations can be used in addition to or without the benefit of pulse shaping. Otherwise, the control system and apparatus are the same as discussed herein.

[0048]   The real time learning feedback method and computer software will now be described in greater detail with regard to Figures 10A and 10B.   This method and software are employed to statistically optimize the repetitive identification of molecularly complex and unknown samples or specimens in a highly automated and relatively quick manner.   The data that is obtained in the mass spectrometer for each laser pulse consists of a two-dimensional array of numbers which are stored in random access memory of the personal computer.   The first column of the array contains arrival times for the ions, when the data is obtained from the time-of-flight

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mass spectrometer. Equivalent numbers can be obtained from different mass spectrometry units such as quadrupole or ion-cyclotron spectrometers. This numbers can be converted to a mass assuming a charge for the species. The second number corresponds to the intensity (RI) of signal at that particular arrival time. This number

5   correlates with the probability of detecting a charge species with that mass (see, for example, Figure 8). The data acquisition involves collecting a number of data sets and then calculating the sum; this action is performed by the personal computer. The target is an ideal mass spectrum defined by the system user. The target can be the maximization or minimization of a signal at a particular single mass, or a number of

10   masses with or without background suppression.

[0049]   Each pulse shape is defined by a two-dimensional array of numbers which are stored in random access memory of the personal computer or otherwise accessible on a read only basis by the personal computer from a disk or the like. The length of each column determines the resolution with which the spectrum of the laser

15   pulse is being sculpted. For a liquid spatial-light modulator, the number of pixels typically determines this number. The first column of numbers determines the amplitude transmission coefficient for every pixel. The second column determines the phase delay for every pixel. The entire array of amplitudes and phases determines the final shape of the output pulse. The shortest pulse is pre-defined as

20   the shortest duration possible for the laser system will the longest pulse is pre-defined as the longest pulse that can be made with the pulse shaper. A two-pulse combination is pre-defined as the combination of an unshaped pulse with a shaped

pulse. Pre-defined ultraviolet or infrared pulses at 400 or 800 nm, for example, can be used.

[0050] For the first iteration, the computer generates a number of different seed pulsed shapes with a pre-determined resolution using a random number generator. For each pulse shape, a number of mass spectra (the number of averages) are summed. The number of averages is determined by the reproducibility of the data obtained. The resulting average data is compared with the target and a success value is calculated. It is expected as the optimum pulse shape is achieved, the number of averages can be reduced because of the higher efficiency of these pulses. A number of successor pulses are chosen based on their success value and the rest of the pulse shapes are discarded. The best one is stored as the optimum. The number of "more iterations" is reset to zero; this number keeps track of how often the optimum pulse is redefined and is used to determine convergence. The success value is a number between zero and one that is assigned to each average set; this number quantifies how well the average data set approximates the target and can be obtained, for example, from a sum of the differences or a sum of the differences squared. Step A, inputting/defining the target value based on the mass, the percent of mass and minimum ion count level is very important as one wants to define regions in the spectrum to be maximized and/or minimized. This also assists in reducing background noise or interference from the mass spectrometer detections. Step B, inputting/defining the number of shots to average for each seat pulse can be set, for example, at 1000 laser beam shots for a single sample without significant

degradation; this is highly advantageous over the typical 100 or less maximum shots traditionally used with prior nanosecond lasers before the specimen is unusable.

[0051] The adjustment and focusing of the shaped beam onto the sample in step J, can be performed manually or automatically by the personal computer. For example, a relatively weak diode laser, having the same wavelength as the main femtosecond laser and following the same path, can be used with a CCD camera to aim and focus the main shaped laser beam onto the sample in an automated and computer controlled, real time feedback manner. Furthermore, step L allows for a setting of delayed pulse extraction within the mass spectrometer. The calculation, comparison, and determination steps, such as those of steps N through V, are all conducted in an automatic manner within the microprocessor of the personal computer.

[0052] Step R allows the microprocessor to determine the best pulse shape with the highest success value and store it as the optimum value in the random access memory of the central processing unit. The computer will then pick approximately the ten percent best pulse shapes based on the highest success values and then reset the discarded values and automatically generate new laser pulse shapes in step V for subsequent testing iterations on the same specimen. The generation of new seed pulses is important for the success of the feedback method. The goal is to arrive at the optimum pulse in the shortest number of iterations making sure that one has searched the entire range of parameters, the global maximum. The "cost functional" refers to the statistical pressure that is placed on the optimum

pulse shape in order to simplify it. For example, once an optical pulse shape or other characteristic is found, it may be important to determine how sensitive the outcome is to each of its amplitude and phase components. Perhaps a much lower resolution can produce the results. The simpler the pulse the easier it is to reproduce and interpret the results in terms of physical concepts. For certain cases, the shape can be simple enough that it can be prepared without a shaper which would allow for a less expensive alternative to the preferred pulse shaping; for example, a combination of two or three different 800 nm pulses, or a combination of infra-red and ultra-violet pulses could be employed as a modified or optimized pulse. Once statistical convergence has been determined by the personal computer, then the test is complete by determining the optimum pulse characteristics (whether they be pulse shape, pulse duration or any other such variable laser beam characteristic) for the corresponding and now post-test identified specimen.

[0053] The time scale of some of the processes that occur during MALDI may be longer than the femtosecond pulses. In a first variation, the pulse shaper can be used to produce pulse sequences up to ten picoseconds apart. Optical delay lines can be used to increase this time delay in the nanosecond range if needed. In a second variation, the wavelength of the pulses being shaped is 800 nm. A second harmonic crystal is all that is needed to convert the wavelength to 400 nm, however, the shaper is capable of regulating the energy delivered to the sample without changing the carrier frequency (wavelength) of the laser.

[0054] The sensitivity and flexibility gained should make this unit cost effective, especially if in-source selective bond cleavage is achieved. Another variation, used to further minimize cost, provides that optimal pulse shapes may be synthesized from a combination of less expensive laser sources.

5   **[0055]** The concern of missing the MALDI crystal by using a sharply focused laser, usually observed when a weak and inexpensive laser source is used, can be overcome with the present system since it is able to use focal spots as large as a millimeter. The peak intensity of the laser will exceed 10^{11} W/cm^2, the ionization threshold due to multiphoton excitation, using 0.1 mJ per pulse focused on a 1 mm

10   diameter spot.

Protein Sequencing

[0056] Laser desorption mass spectrometry can be employed with the present invention for identification and protein sequencing. This is significantly enhanced and made possible by the ultra-fast laser pulses and learning feedback

15   system used. The matrix has been shown to enhance the yield of charged protein for analysis by MS detection. The matrix:phosphor diester backbone interaction has been shown to play an important role. The use of liquid matrices such as glycerol and lactic acid for IR-MALDI may bring some additional flexibility to sample preparation and delivery to the MALDI instrument. The "Ladder Sequencing" method

20   involves a partial Edman degradation with phenyl isothiocyannate and using phenyl isoccyanate as a terminating agent. Partial enzymatic hydrolysis of polypeptides using trypsin is another strategy for protein sequencing. Trypsin digestion attaches

only bonds in which the carboxyl group is contributed by either a lysine or an arginine residue. Analysis of metastable species in MALDI-PSD using a reflectron TOF spectrometer leads to valuable structural information. The introduction of 'delayed extraction' in MALDI allows improved resolution, suppression of matrix background, reduction of chemical noise, and minimization of the effect of laser intensity on performance.

[0057] MALDI is a soft ionization technique which produces protonated molecules that undergo very little or no subsequent fragmentation due to the low amount of energy imparted during the ionization process. Therefore, MALDI can be used to analyze mixtures of peptides because the mass spectrum of one peptide is unlikely to overlap with the spectrum of another. Ideally, cleavage of the ionized peptide at each peptide bond would provide a mass spectrum that could be interpreted, using knowledge of the masses of the amino acid residues, to deduce the sequence. However, as conceptually illustrated in Figure 11, cleavage on either side of the I-carbon is also possible to give fragment ions, which, while diagnostically useful, also complicate the spectrum. It is also noteworthy that cleavage at any designated bond can generate either an N-terminal ion (a, b, c) or a C-terminal ion (x, y, z), the predominance of which for a protonated peptide (MH$^+$) depends on the locus of the more basic residues. In reality, the fragmentation process is more complicated than suggested in Figure 11; for example, creation of a y-ion involves hydrogen transfer from the N-terminal side of the peptide bond and retention of the ionizing proton. In addition, there can be fragmentation of the side chain on certain

residues; for example, fragmentation involving cleavage at the ϑ-carbon of leucine and isoleucine generates w-ions, which distinguish these two isomeric residues.

[0058] Recognizing the ion types as represented by the appearance of peaks in the mass spectrum is not critical, as most strategies for interpretation, especially those using an algorithm, involve an iterative computational approach. However, the beginning of a C-terminal series of fragments can be distinguished from the start of an N-terminal series. The largest b-ion will be represented by a peak at high m/z value that differs from that representing MH$^+$ by a number of mass units equal to the sum of the mass of an amino acid residue plus the mass of water due to expulsion of the C-terminal residue, which contains the hydroxyl group. On the other hand, the largest y-ion is represented at a high m/z value by a peak differing from that for MH$^+$ by a number of mass units equal to only the mass of an amino acid residue.

[0059] In principle, the sequence of a peptide is deduced from a mass spectrum in which a complete series of any given ion type are represented. In practice, however, a complete series of any one type is rarely observed, but in fortunate situations, overlapping patterns of two or more incomplete series may give complete sequence information. Ideally, one would prefer to observe complementary information from series of N-terminal and C-terminal fragment ions to bolster confidence in the analysis.

[0060] Consider the MALDI-PSD mass spectrum shown in Figure 8 as an unknown. It can be assumed at the outset that the major peak at m/z 574 Da

represents the protonated molecule, which was the precursor ion selected for PSD. The protonated molecules fragment during the PSD process and degrade into fragment ions represented by the peaks at lower m/z shown in Figure 8. The procedure for analysis or data interpretation consists of merely examining the mass difference between each of the fragment ion peaks and the peak representing the protonated molecule. The goal is to find a fragment ion peak that differs in mass from the protonated molecule peak by either a residue mass or a residue mass plus water. A peak at a mass-to-charge (m/z) value that differs from the protonated molecule peak by the mass of a residue mass plus water corresponds to the amino acid that was located at the C-terminus of the original peptide.

[0061] In considering the mass spectrum in Figure 8, the peak at m/z 425 corresponds to the loss of 149 Da, which corresponds to the mass residue of methionine and water; i.e., 149 = 131 (the residue mass in methionine) + 18. The observation of the peak at m/z 425 suggest that methionine was expelled from the C-terminus to form a b ion at m/z 425. Having recognized the largest b ion in the mass spectrum by its peak at m/z 425, the goal is to identify the next smaller b ion, namely one that also has lost the second amino acid residue from the C-terminus to form the second b ion. The peak at m/z 278 represents a mass difference from 425 that corresponds to only the residue mass of an amino acid. That is, the mass difference between 425 and 278 is 147 Da, which is the residue mass of phenylalaninne. This suggests that the second amino acid in the sequence from the amino terminus is phenylalanine: -Phe-Met.

[0062]   Continuing with the b series, the peak at m/z 221 is 57u lower than 278. This mass difference corresponds to the residue mass of glycine: -Gly-Phe-Met. This observation suggests that the third amino acid from the C-terminus is a glycine residue. Finally, it can be seen that there are no other peaks observed below

5   m/z 221 that correspond to the loss of a residue mass from 221; the search for further b ions come to a halt. At this point, the peak represents the intact molecule (the peak at m/z 574 which is the protonated peptide) and an examination of fragment ion peaks that differing in mass from 574 by exactly a residue mass to try to recognize the beginning of the y series of ions, is pursued. Upon reconsideration of

10   this mass spectrum, one notices that the peak at m/z 411 is 163 lower than the peak at m/z 574. This corresponds to the residue mass of tyrosine. Residue loss from the protonated molecule suggests that tyrosine is the amino acid located at the amino terminus of the peptide.

[0063]   With the focus now on m/z 411 as a reference point, notice that the

15   peak at m/z 354 is 57 Da lower, a mass which corresponds to the residue of glycine. The observation of peak at m/z 354 suggests that the second amino acid from the N-terminus is a glycine residue, giving the partial sequence: Tyr-Gly-. Using the peak at m/z 354 as a reference point, the peak at m/z 297 is 57 Da lower than m/z 354 and suggests the expulsion of another residue of glycine in the formation of this ion.

20   These observations suggest that the third amino acid residue is also a glycine residue in sequence from the N-terminus. Continuing with the peak at m/z 297 as a reference point, the peak at m/z 150 is 147 Da lower, suggesting expulsion of a

phenylalanine residue or that phenylalanine is the fourth amino acid from the N-terminus, giving the partial sequence: Try-Gly-Gly-Phe-. Since there are no other peaks at lower m/z that differ from m/z 150 by a residue mass of an amino acid, recognition of the sequence comes to a halt.

[0064]   Having suggested possibilities for amino acid sequences from either terminus of the peptide, these two suggested partial sequences are overlaid to compose a complete sequence. For example, the data reviewed above suggest that the sequence at the C-terminus is: -Gly-Phe-Met; whereas another series of data suggested that the series of amino acids at amino terminus was: Try-Gly-Gly-Phe-. Note that each of these two partial sequences shows the C-terminus of a glycine residue connected to the amino terminus of a phenylalanine residue. These two residues must be a redundant observation in the two sequences, and they can be overlaid at that point. This would give an overall sequence starting from the amino terminus of: Try-Gly-Gly-Phe-Met.

[0065]   If the residue masses of the postulated amino acids in the complete sequence are summed, 555 Da will be the obtained value. Add 18 Da to this sum for the hydrogen at the amino terminus and the hydroxyl at the C-terminus. Then add 1 Da for the proton on the protonated molecule. This will give a total of 574 Da for the expected mass of the protonatetd molecule, in perfect agreement with the observed peak at m/z 574, giving credence to the suggested amino acid composition implicit in the sequence Y-G-G-F-M.

[0066]   The protein sequencing can be conducted by use of the present invention with or without use of a matrix. The use of an ultrafast, femtosecond laser is envisioned to minimize any destructive burning of the specimen, thereby potentially rendering use of an expensive and time-consuming matrix as unnecessary. Without a matrix (herein, also known as having "isolated molecules"), the identification and sequencing of the protein is simplified since the matrix characteristics do not have to be accounted for and filtered out of the calculations. A femtosecond laser in the range of approximately 20 femtosecond duration pulses allows for localization of the energy based on the speed of the pulse and the ability to quickly shape the phase and amplitude modulation of the pulse. Furthermore, the specimen fragmentation is primarily due to laser cleavage rather than enzyme or chemical cleaving. This is ideally suitable for insoluble proteins, glycocylated proteins which have been linked to cancer, (including the selective cleavage of the associated oligosacharides) direct protein analysis from silicate substrates, direct analysis of PAGE gels, direct sampling of membrane proteins from intact cells and bacteria, the direct sampling of genetically modified agricultural produce (such as grains), and even human matter such as hair, fingernails and fingerprints.

[0067]   The personal computer employs a method and software for protein sequencing as follows. The foundation of this method is based on the fact that there are only 20 amino acids and that their masses are well known. First, the computer determines the molecular weight of the intact proteins specimen. This requires the generation of a single high-mass peak and minimization of the low weight

background. Secondly, the computer automatically finds peaks that are an integer number of amino acids smaller than the parent protein; a laser beam pulse shape that causes some fragmentation can be employed. Thirdly, this procedure is continued from high to low masses. Finally, confirmation of results can be automatically obtained by a statistical optimization method (such as that previously described for the MALDI process) that attempts to optimize a given mass; the success of this optimization will depend on whether that fragment of the protein has an integer number of amino acids. Automatic adjustment for the N or C terminus is also automatically adjusted for by the computer as previously explained. Alternately, each single amino acid could be separately searched for. Thus, the present invention control system and apparatus is ideally suited for analyzing, identifying, sequencing and severing complex multi-molecular specimens in a highly automated manner.

Selective Bond Cleavage

[0068] The ultra-fast laser of the present invention is used to enhance in-source photochemistry and fragmentation, however, random fragmentation would not be as useful as selective bond cleavage. Furthermore, selective peptide bond cleavage would be ideal for protein sequencing. Cleavage of amino acid side chains may be of value for de novo sequencing because it would allow a determination of the presence or absence of certain amino acids. Similarly, selective cleavage of phosphate groups, oligosacharides and other post-translational modifications would be equally valuable. The ideal, of course, would be to achieve peptide bond

cleavage without loss of side chains or other appended groups. This would allow, for example, to determine phosphorylation sites.

[0069] It is envisioned that selective bond cleavage can be realized when using shaped pulses that are capable of localizing the energy in a time scale that is short enough to prevent total energy randomization. For example, the protonated molecule of bradykinin potentiator C, as produced by MALDI, fragments poorly during PSD, and does not produce a suitable spectrum from which one could deduce the amino acid sequence. Thus, this 11-residue peptide is ideal for this application. Selective laser bond cleavage may have additional application as a synthetic route to specific products.

Fixed Shape Pulse Shaping Apparatus

[0070] A second preferred embodiment of a laser excitation apparatus 121 is showing in Figure 12. Apparatus 121 includes a femtosecond laser 123, an upstream prism 125, a downstream prism 133, a pulse shaping mirror 129 at the Fourier plane, an offset mirror 131, and a receiver or targeted specimen 135. Upstream prism 125 initially acts to disperse the colors of the emitted laser beam pulse while downstream prism 133 serves to redirect this dispersed laser beam pulse toward pulse shaping mirror 129. Pulse shaping mirror 129 has a predetermined or fixed pulse shaping surface, such as a set of 800 nm wavelength sine curves, formed or machined therein. The physical characteristic or shape of the actual pulse shaping surface is predetermined through optimization experimentation for the intended use and intended laser beam input by use of a learning program such as that disclosed in

the first preferred embodiment. After the desired mirror surface shape is known for the intended use, the less expensive, fixed shape mirror 129, or an alternate fixed pulse shaping optic, can be employed to reduce equipment costs for actual production systems. Also, the computer and optimization program are not required for these types of known set up and known specimen applications after the initial determination is conducted.

[0071]   The passive pulse shaping mirror 129 thereby reshapes the laser beam pulse shape characteristic, reflects it back through the same prisms in reverse order, and in an offset or time-delayed manner. The position and orientation of mirror 129 alters the final characteristics of the shaped pulse. Thus, a computer controlled automatic actuator can move or change the position or orientation of mirror 129, based on an algorithm, such as shaping the pulse with the cosine portion of the sine wave shape. Offset mirror 131 subsequently reflects the shaped laser beam toward the receiver, which can be a mass spectrometer, fiber optic sensor/switch, or a targeted specimen.

[0072]   It is alternately envisioned that an in-line optical apparatus can be used, such as that disclosed with the first preferred embodiment, however, the pulse shaper at the Fourier plane would be replaced by a passive mask having a transmissive optic with a predetermined coefficient of refraction, or a polarizing-type sine mask on a transparent substrate. Also, a polymer-doped glass or blend of polymer sheets that are capable of retarding the phase of the laser beam pulse wave

or otherwise varying a wavelength, timing or shaping characteristic of same can be employed.

[0073]   Alternately, certain optics can be used such as a backside coated, chirped mirror having multiple dichroic layers, which would be satisfactory for pulse shaping without dispersive optics and without the need for a Fourier plane. An acceptable chirped mirror is disclosed in Matuschek, et al, "Back-side-coated Chirped Mirrors with Ultra-smooth Broadband Dispersion Characteristics," *Applied Physics B*, pp. 509-522 (2000). A negative dispersion mirror from CVI Laser Corp., part no. TNM2-735-835-1037 is another suitable example. A rotatable wheel having multiple different chirped mirrors, each with specific pulse shaping characteristics, can also be used to provide a discrete number of predetermined shaped pulses.

Optical Coherence Tomography

[0074]   A third preferred embodiment of the present invention uses an apparatus 221 for laser excitation or ionization with Optical Coherence Tomography ("OCT"). In general, Figure 17 illustrates the OCT application of apparatus 221 wherein there is a femtosecond laser 223, a laser beam shaper 229, a human or animal tissue specimen 241, an optical gate 251 and an image 253. Laser 223 emits a laser beam pulse shorter than 1 picosecond. Shaper 229 is made of three parts; two dispersive elements 255 which sandwich a phase mask element 257. Shaper 229 essentially prevents multiphoton excitation which can damage the person's or animal's DNA, as will be discussed in more detail as follows. An unshaped laser beam pulse is used to gate the ballistic photons to render the image for tomography

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use. Optical gating can be accomplished by up-conversion in a frequency doubling crystal or with a kerr-gate in liquid carbon disulphide. The construction of apparatus 221 as illustrated supposes transmission imaging; the same end result can alternately be accomplished with back scattered imaging. Image 253 could be viewed like an x-ray-type image of the internal organs of the human or animal specimen but without harmful three photon exposure. The use of the shaped pulse in OCT provides for an increase in laser intensity for better imaging while preventing the damaging effects caused by multiphoton excitation of healthy tissue.

Photodynamic Therapy

[0075]   A fourth preferred embodiment of the present invention uses an apparatus also shown as 221 for laser excitation or ionization with photodynamic therapy ("PDT"). In general, Figure 17 also illustrates the PDT application of apparatus 221, but optical gate 251 and image 253 are not being required. Shaper 229 allows two photon excitation but essentially prevents three photon excitation. Shaper 229 enhances the laser induced activity of a therapeutic agent which prevents damage of healthy tissue. Use of laser beam pulse shaping of the present invention should provide superior control and results for PDT applications as compared to those practically possible with conventional methods as disclosed, for example, in U.S. Patent No. 6,042,603 entitled "Method for Improved Selectivity in Photo-Activation of Molecular Agents" which issued to Fisher et al. on March 28, 2000, and is incorporated by reference herein. Alternately, the pulse shaper can be

tuned to target cancerous cells for multiphoton gene therapy or destruction, with or without the presence of a therapeutic agent, without damaging healthy tissue.

Control of Nonlinear Optical Processes

[0076] As applied to all of the applications herein, selective control of one and multiphoton processes in large molecules, including proteins, is possible using simple pulse shaping. The results show an extraordinary level of control that is robust and sample independent, with contrast ratios near two orders of magnitude (clearly visible with the naked eye). Such large contrast ratios allow for more precise cancellation control of undesired photons and other laser beam characteristics, such that nonlinear transitions induced by each pulse are controlled. Because simple phase functions can be incorporated into a passive optical component such as mirror 129 (see Figure 12), these applications do not require the complexity and expense of computer controlled pulse shapers after initial set up, although systems such as in Figure 1 can still be employed.

[0077] The underlying concept of the apparatus and associated method are shown in Figures 13A – 13C. Multiphoton transitions are optimized when the central bandwidth of the laser pulse ω_0, is some fraction (half for two-photons, a third for three-photons, etc.) of the total energy of the transition as illustrated in Figures 13A and 13C. For ultrafast pulses, when the bandwidth is large, different frequency components ($\omega_0 \pm \Omega$) of the pulse can interfere, thereby reducing the probability for multiphoton excitation. Referring to Figure 13B, the spectrum of the ultrafast laser pulse with amplitude $A(\Omega)$ is plotted as a function of detuning from the central

frequency. A phase mask $\varphi(\Omega)$ can be imprinted on the pulse such that the phase of each frequency component Ω acquires a specific value. The effect of pulse shaping on the probability for two-photon absorption ("2PA") can be calculated as follows:

$$P_{2PA} \propto \left| \int_{-\infty}^{\infty} A(\Omega)A(-\Omega)\exp[i\{\varphi(\Omega)+\varphi(-\Omega)\}]d\Omega \right|^2$$

[4]

5   and for three-photon absorption ("3PA"), a similar formula can be derived as follows:

$$P_{3PA} \propto \left| \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} A(\Omega_1)A(\Omega_2)A(-\Omega_1-\Omega_2)\exp[i\{\varphi(\Omega_1)+\varphi(\Omega_2)+\varphi(-\Omega_1-\Omega_2)\}]d\Omega_1 d\Omega_2 \right|^2$$

[5]

where amplitudes and phases are introduced for two different detuning values Ω_1 and Ω_2, as shown in Figure 13C. One photon transitions are not affected by the phase of the pulses, however, one photon processes are difficult to selectively achieve at high

10   photon flux due to the onset of multiphoton processes.

[0078]   A schematic representation of two photon and three photon induced fluorescence is illustrated in Figures 13A and 13B, respectively. The vertical arrows represent ultrafast pulses that induce the two and three photon transitions. Because of their broad bandwidth, ultrafast pulses contain photons that are detuned from the

15   central wavelength ω_0 by an amount Ω. Referring again to Figure 13C, ultrafast laser pulses are shaped using a sine function phase mask across the pulse spectrum underlying the dashed curve while the structures of the chromophores are also shown.

Example 1

[0079]   The experiments in all of the following example were carried out using an amplified titanium sapphire laser producing 50 fs pulses. The pulses were shaped using a spatial light modulator ("SLM") at the Fourier plane of a zero-dispersion two grating arrangement. The two independent modulator plates, based on liquid crystal technology in the SLM (128 pixels each), were calibrated so that only phase delays were introduced without changes to the output spectrum, intensity, and polarization. The shaped pulses centered at 809 nm were characterized by second harmonic generation frequency resolved optical gating. When all phases were set to zero, laser pulses were near transform limited. Unless indicated otherwise, measurements were made with pulse energies of 0.4 μJ/pulse at the sample. Experiments were carried out by setting the phase function equal to a sinusoid, as shown in Figure 13B, in the 779-839 nm spectral range. Emission from one photon or multiphoton induced processes from various samples was measured as a function of δ, the phase shift of the mask across the spectrum. The maximum phase advancement or retardation was 1.5π.

[0080]   Equations 4 and 5 can be used to calculate the expected signal for two and three photon processes as a function of δ. These calculations are graphed in Figures 14A – 14H for sinusoidal phase functions having a half (Figures 14A and 14B) or a full (Figures 14C and 14D) period across the entire phase mask. The calculated probability for two photon and three photon transitions peaks at half integer values of π in Figures 14A and 14B, while the calculated probability for two photon and three photon transitions peaks at integer values of π in Figures 14C and

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14D. The shape of the phase function, where maxima and minima in the probability are achieved, is indicated as inserts.

[0081]   Experimental data were obtained with the phase functions used for the calculations in Figures 14A - 14D. In these experiments, the two and three photon emission from large organic molecules is detected as a function of δ. Although the model described by equations 4 and 5 assumes two level systems, Figures 14E -14H experimentally demonstrate that this principle can be applied to complex systems having a manifold of vibrational states broadened by the presence of a solvent. It is noteworthy that the peaks and valleys predicted by equations 4 and 5 are observed in the experimental data; essentially, the intensity maxima are found when the phase function is antisymmetric with respect to the central wavelength of the pulse and minima when it is symmetric.

[0082]   More specifically, theoretical and experimental phase-mask control of two and three photon induced fluorescence is shown in Figures 14A – 14H. Equations 4 and 5 predict that as the phase mask is translated by an amount δ, the probability of two ("P_{2PA}") and three photon transitions ("P_{3PA}") is modulated, as illustrated in Figures 14A – 14D, for a half period sine mask (Figures 14A and 14B) and a full period sine mask (Figures 14C and 14D). Transform limited pulses yield a maximum value of 1. The small inserts in Figures 14A and 14C display the phase function at specific positions where maximum and minimum values of fluorescence take place (Figures 14E – 14H) wherein experimental two and three photon laser induced fluorescence measured for Coumarin and Stilbene, respectively, as a

function of phase mask position δ are shown. The phase masks used for these experiments were the same as those used in the calculations. Thus, the pulse shaping masks can be predetermined or fixed in shape based on calculations, experiments or learning program values for known equipment and known specimens.

5   Example 2

[0083]   Experimental results for various samples obtained with a full-period sinusoidal phase mask are shown in Figures 15A – 15G. Figure 15A shows one photon laser induced fluorescence ("1PLIF") of IR144 observed at 842 nm as a function of phase mask position. This measurement was made with 0.3 nJ/pulse to

10   avoid nonlinear processes at the specimen. It is noteworthy that one photon process in the weak field regime show no dependence on phase shaping. Figure 15B shows results for the two photon laser induced fluorescence ("2PLIF") from Coumarin collected at 500 nm. The data in Figure 15C shows the dependence of 2PLIF in recombinant green fluorescent protein ("rGFP") detected at 505 nm. The data in

15   Figure 15D corresponds to the intensity of the second harmonic generation ("SHG") signal at 405 nm from a 0.3mm β-barium borate crystal. The maximum and minimum signal for SHG coincides with that observed for 2PLIF but is not identical.

[0084]   With reference to Figure 15E, the dependence of three photon laser induced fluorescence ("3PLIF") from Trans-Stilbene is illustrated. The signal was

20   collected at 350 nm as a function of δ. In this case, the maximum contrast (max:min) is measured to be 60:1. The data in Figure 15F corresponds to the 3PLIF from Tryptophan residues in Con A, collected at 350 nm. In 3PLIF the maximum

fluorescence signal is less than that obtained for transform limited pulses (when all the phases in the mask are set equal to zero), but the overall contrast ratio over the three-photon excitation is excellent, approaching two orders of magnitude. The data in Figure 15G corresponds to the continuum generation response (a nonlinear self-frequency modulation process yielding white light pulses) from a 3 mm slab of quartz detected at 600 nm.

[0085]   More specifically, Figures 15A – 15G demonstrate the experimental measurements of one and multi-photon emission obtained as a function of phase mask position δ. In all cases, the phase mask is a full period sine function. The signal measured with transform limited pulses is unity. The contrast ratio (max:min) is given in the upper right corner of each of the experimental plots. Here we find that the higher the order of the optical nonlinearity, the greater the contrast that we observe, therefore discrimination among different order processes is possible. In other words, the higher the order, the greater the photons, which makes it easier for photon cancellation. Also, the greater the contrast ratio, the more the background noise is filtered out.

Example 3

[0086]   Figure 16A presents the maximum discrimination between linear and nonlinear response observed for intense pulses (0.5 μJ/pulse). Separate detectors simultaneously collected the 1PLIF from IR144 solution and a portion of the continuum output. Maximum and minimum contrast ratios of $>10^3$:1 and 1:0.6 were obtained for one photon process versus continuum, respectively, as shown in Figures

16A and 16B. This control is extremely valuable when one is interested in linear processes under high-flux conditions, like in laser microscopy or in optical fiber communications. Using the simple phase function discussed earlier, particular windows of opportunity to control second versus higher order processes can be employed as demonstrated in Figures 16C and 16D. For certain values of δ, continuum generation even for relatively high intensity laser pulses (~1 μJ/pulse) can be completely suppressed. Figures 16C and 16D show that maximum and minimum contrast ratios of >10^3:1 and 1:4 were obtained for 2PLIF versus continuum, respectively.

[0087] Two photon transitions can be achieved while suppressing three photon processes for use in two photon microscopy or in two photon PDT. This type of control is much more difficult because once multiphoton transitions take place it is very difficult to stop at a particular order. A mixture of Coumarin and Fluoranthene were prepared to explore control of 2PLIF versus 3PLIF. Because fluorescence from these two molecules overlaps the same spectral region, the separation between the two signals was achieved by temporal gating. Coumarin fluorescence was detected at 495 nm during the first 20 ns, while fluoranthene fluorescence was detected at 460 nm with a gate that opened 40 ns after the initial rise and extended for 120 ns. Maximum and minimum contrast ratios of 1.4:1 and 1:2.2 were obtained for 2PLIF versus 3PLIF, respectively, as presented in Figures 16E and 16F. The contrast data presented in Figures 16A – 16F were obtained when transform limited pulses yielded equal intensities for the processes. Better contrast can be obtained using additional

pulse shaping as described in the following section, especially as the multiphoton processes are detuned from resonance.

Predetermined Pulse Shaping and Phase Control of Multiphoton Processes

[0088] The present invention can utilize the presently invented phenomenon of "Multiphoton Intrapulse Interference" as optimized for large molecules, proteins, and other condensed phase materials, through a combination of: (a) a chirped mask pulse shaper; and (b) a smooth function of phase versus frequency for the mask pulse shaper. The following formulas provide a predictive advantage for finding appropriate phase masks instead of using a learning program. The probability of two photon transitions can be calculated as follows for any given pulse shape:

For an electric field with a carrier frequency ω_0 and a slow amplitude $E_0(t)$,

$$[6] \qquad E(t) = E_0(t)e^{-i\omega_0 t} \qquad \text{and} \qquad E_0(t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} F_0(\Omega)e^{-i\Omega t} d\Omega$$

where the Fourier image $F_0(\Omega)$ around carrier frequency $\Omega = \omega - \omega_0$ can be written as:

$$[7] \qquad F_0(\Omega) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} E_0(t)e^{i\Omega t} dt,$$

the amplitude of two photon transition at resonance frequency ω is:

$$[8] \qquad A_2(\omega) \propto \int_{-\infty}^{\infty} E(t)^2 e^{i\omega t} dt = \int_{-\infty}^{\infty} E_0(t)^2 e^{i(\omega - 2\omega_0)t} dt = \int_{-\infty}^{\infty} E_0(t)^2 e^{i\Delta t} dt,$$

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where detuning $\Delta = \omega - 2\omega_0$, the probability of two photon transition is:

[9] $P_2(\omega) = |A_2(\omega)|^2$.

[0089]   Furthermore, the Fourier image of convolution is the product between Fourier images

[10] $T(f*g) = (Tf)(Tg)$

5   where convolution (*, function from Δ) of two functions (f) and (g) is:

[11] $f*g = \dfrac{1}{\sqrt{2\pi}} \displaystyle\int_{-\infty}^{\infty} f(\Omega)g(\Delta-\Omega)d\Omega.$

Direct (T, function from Ω) and inverse (T^{-1}, function from t) Fourier images are

[12] $T(f) = \dfrac{1}{\sqrt{2\pi}} \displaystyle\int_{-\infty}^{\infty} f(t)e^{i\Omega t}dt$ and $T^{-1}(f) = \dfrac{1}{\sqrt{2\pi}} \displaystyle\int_{-\infty}^{\infty} f(\Omega)e^{-i\Omega t}d\Omega.$

Additionally, the relation between direct and reverse transforms is:

[13] $T^{-1}T(f) = TT^{-1}(f) = f.$

Thus, using the inverse transform, the formula can be written as:

[14] $f*g = T^{-1}T(f*g) = T^{-1}[(Tf)(Tg)]$ or

formula [14] in integral form is as follows:

[15] $\displaystyle\int_{-\infty}^{\infty} f(\Omega)g(\Delta-\Omega)d\Omega = \int_{-\infty}^{\infty} e^{i\Delta t}[(\dfrac{1}{\sqrt{2\pi}}\int_{-\infty}^{\infty} f(\Omega)e^{-i\Omega t}d\Omega)(\dfrac{1}{\sqrt{2\pi}}\int_{-\infty}^{\infty} g(\Omega)e^{-i\Omega t}d\Omega)]dt.$

10

[0090] The time-frequency transformation can be calculated. Using the spectral presentation of formula [7] and convolution theorem of formula [15], formula [8] can be rewritten to obtain the formula for two photon transitions as follows:

[16] $\quad A_2(\Delta) \propto \int_{-\infty}^{\infty} E_0(t)^2 e^{i\Delta t} dt \propto \int_{-\infty}^{\infty} F_0(\Omega) F_0(\Delta-\Omega) d\Omega$

This expression provides the two photon absorption amplitude given the spectrum of

5   the laser pulse $F_0(\Omega)$ and the detuned spectrum of the $F_0(\Delta-\Omega)$ that depends on the absorption spectrum of the sample.

[0091] The probability of three photon transitions can be subsequently calculated.

The complex amplitude of transition is:

[17] $\quad A_3(\omega) \propto \int_{-\infty}^{\infty} E(t)^3 e^{i\omega t} dt = \int_{-\infty}^{\infty} E_0(t)^3 e^{i\Delta t} dt,$

10   where detuning $\Delta=\omega-3\omega_0$. Using the reverse Fourier presentation for the fields of formula [6], formula [17] can be rewritten as:

[18] $\quad A_3(\omega) \propto \int_{-\infty}^{\infty} e^{i\Delta t}[\int_{-\infty}^{\infty} F_0(\Omega) e^{-i\Omega t} d\Omega \int_{-\infty}^{\infty} F_0(\Omega) e^{-i\Omega t} d\Omega \int_{-\infty}^{\infty} F_0(\Omega) e^{-i\Omega t} d\Omega] dt$

Next, equation [18] can be rewritten using a new function $G(\Omega)$

[19] $\quad A_3(\omega) \propto \int\limits_{-\infty}^{\infty} e^{i\Delta t} [\int\limits_{-\infty}^{\infty} F_0(\Omega_1)e^{-i\Omega_1 t}d\Omega_1 \int\limits_{-\infty}^{\infty} G(\Omega_1)e^{-i\Omega_1 t}d\Omega]dt,$

where $G(\Omega_1)$ is defined as the kernel of the integral

[20] $\quad \int\limits_{-\infty}^{\infty} G(\Omega_1)e^{-i\Omega_1 t}d\Omega_1 = \int\limits_{-\infty}^{\infty} F_0(\Omega_1)e^{-i\Omega_1 t}d\Omega_1 \int\limits_{-\infty}^{\infty} F_0(\Omega_1)e^{-i\Omega_1 t}d\Omega_1,$ and

using the convolution formula [15], the following formula is obtained:

[21] $\quad A_3(\omega) \propto \int\limits_{-\infty}^{\infty} F_0(\Omega_1)G(\Delta-\Omega_1)d\Omega_1.$

[0092] The Fourier image of the Reverse Fourier image of equation [20] defines the intermediate function using relationship of equation [13] and the integral

5 form of the convolution theorem expressed in formula [15] as:

[22]
$$G(\Delta-\Omega_1) \propto \int\limits_{-\infty}^{\infty} e^{i(\Delta-\Omega_1)t} [\int\limits_{-\infty}^{\infty} F_0(\Omega_2)e^{-i\Omega_2 t}d\Omega_2 \int\limits_{-\infty}^{\infty} F_0(\Omega_2)e^{-i\Omega_2 t}d\Omega_2]dt =$$

$$\int\limits_{-\infty}^{\infty} F_0(\Omega_2)F_0(\Delta-\Omega_1-\Omega_2)d\Omega_2$$

[0093] The final formula for the detuned $\Delta=\omega-3\omega_0$ three photon transition is obtained by using equations [21] and [22] after changing the order of integration:

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[23] $A_3(\Delta) \propto \int\limits_{-\infty}^{\infty} E_0(t)^3 e^{i\Delta t} dt = \int\limits_{-\infty}^{\infty} ... \int\limits_{-\infty}^{\infty} F_0(\Omega_1)F_0(\Omega_2)F_0(\Delta-\Omega_1-\Omega_2)d\Omega_1 d\Omega_2$

such that the probability is:

[24] $P_3(\omega) = |A_3(\omega)|^2.$

The method described above gave the formula for the n-photon transition by recurrence:

[25] $A_n(\Delta) \propto \int\limits_{-\infty}^{\infty} E_0(t)^n e^{i\Delta t} dt \propto \int\limits_{-\infty}^{\infty} ... \int\limits_{-\infty}^{\infty} F_0(\Omega_1)...F_0(\Omega_{n-1})F_0(\Delta-\Omega_1...-\Omega_{n-1})d\Omega_1...d\Omega_{n-1}$

where detuning is $\Delta=\omega-n\omega_0$. Thus,

[26] $P_n(\omega) \propto |A_n(\omega)|^2.$

5

[0094]   It is also desirable to take into account inhomogeneous broadening (as encountered in solutions and condensed phase materials). The integrated probability for the n-photon transition in molecules with spectral a density $g_n(\omega)$ with amplitude defined by formula [25] is proportional to the weighed average

[27] $P_n = \int\limits_{-\infty}^{\infty} g_n(\omega)|A_n(\omega)|^2 d\omega.$ (1)

10   Normalization for the case of transform limited laser pulse is N_n and

[28] $N_n = \int\limits_{-\infty}^{\infty} g_n(\omega)|A_{TLn}(\omega)|^2 d\omega,$

where

[29] $$A_{TLn}(\omega)= \quad \ldots \int_{-\infty}^{\infty} |F_0(\Omega_1)|\ldots|F_0(\Omega_{n-1})||F_0(\Delta-\Omega_1\ldots-\Omega_{n-1})|d\Omega_1\ldots d\Omega_{n-1}.$$

[0095]   The preceding formulas [6] – [29] give the general result. The following parameters must be defined, however, for a user to define a phase mask that would minimize or maximize a particular multiphoton process. First, the laser pulse spectrum of Figure 18A must be defined. The shorter the pulses (broader spectrum), the better the control. 45 fs pulses have been satisfactorily used but 20 or 10 fs would lead to even better results. The carrier frequency (or center wavelength) must also be defined by availability. Tuning the wavelength of the pulse could enhance certain processes but is not typically required. Secondly, the phase modulator (or alternately, the SLM, deformable mirror, chirped mirror, etc.) should cover the entire pulse spectrum and must be defined. Thirdly, a phase mask definition should be introduced. The simple sine function of Figure 18B works remarkably well, yet other functions that can become symmetric and antisymmetric as a function of their position are also suitable. Fourthly, the addition of positive or negative linear chirp β further enhances the observed control, as expressed in Figure 18C, and should be defined. The phase mask used in the examples presented herein is defined by

$$[30] \qquad \varphi_m(\lambda,\delta) = \varphi_a \sin\left(\frac{\lambda - \lambda_{min}}{\lambda_{max} - \lambda_{min}} \cdot 2\pi N - \delta\right)$$

where δ is the position of the sine function (centering) across the spectrum, ϕ_a is the maximum phase delay, and N_{pixel} is the number of pixels in the SLM, as illustrated in Figure 18B.

[0097] When chirp is added, it can be defined by

$$[31] \qquad \phi_c(\Omega) = \tfrac{1}{2}\,\beta\Omega^2$$

where β is the amount of linear chirp expressed in Figure 18C. Thus, the complete phase mask with chirp is:

$$[32] \qquad \phi(\lambda) = \phi_m(\lambda,\delta) + \phi_c(\lambda).$$

[0098] Figures 19A and 19B show the calculated two and three photon absorption probability using the equation presented and the absorption spectra as calculated by the dotted lines in Figures 20A – 20C. Figure 20C shows the calculated ration two:three photon absorption for the two different combinations of absorption spectra given in Figures 20A and 20B. Accordingly, robust control of multiphoton processes in molecules, proteins and nonlinear optical materials can be achieved through either adaptive, active and self optimizing learning programs and control systems, or through calculated, predetermined or fixed, passive laser beam pulse shaping devices. Therefore, inexpensive fixed phase masks can be designed before the experiment, and even without computer controlled shapers and learning programs, to control the order of multiphoton processes for large, complex molecules, proteins in photodynamic therapy, optical tomography, surgery (such as

laser cutting by five or greater photon wave conveyance to maximize nonlinear energy), and photochemistry control of, for example: (a) photopolymerization (by photon pair switching to seed the process), (b) charge transfer, (c) radical reaction, (d) nucleophelic attack and (e) electrophylic attack.

5   Communications

[0099]   With reference to Figure 21, a fifth preferred embodiment of a laser excitation apparatus 421 of the present invention employs a femtosecond laser 423, an optical fiber 451, a laser beam pulse shaper device 429, a laser beam pulse un-shaper device 453, and a receiver 441 which includes an optical switch or sensor and

10   the related circuitry and electrical control unit.   Laser 423 emits a series of laser beam pulses, each shorter than 1 ps, into the connected fiber 451. Pulse shaper device 429 is of a predetermined mask type with a fixed pulse characteristic varying shape (such as with calculated sine wave surface shapes) and has three elements connected to fiber 451: a dispersive element 455 such as a fiber that incorporates a

15   diffraction grating; a phase mask element 457 that can be made using a doped glass or polymer sheet; and a dispersive element 459, like element 455 but reversed, for accepting spectrally dispersed light and coupling it back to fiber 451.

[00100]   The shaped laser beam pulse is capable of traveling long distances through fiber 451 without suffering nonlinear distortion because of the unique phase

20   function imprinted or formed on shaper device 429. For example, the red color spectrum may be advanced in front of the blue color spectrum in a precise sine manner.   Un-shaper device 453 subsequently reverses the phase changes

introduced by shaper device 429. It is constructed the same as the shaper device but with a different phase mask element 461 that compensates for the pulse characteristic changes made by mask element 457. Alternately, an acousto-optic modulator or transient grating can be used for optical switching through constructive

5   or destructive reference of waves. Shaping and unshaping can also be accomplished by means of a chirped mirror or spectral masks.

[00101] Thus, the present invention's ability to precisely control the laser beam pulse shape or other characteristic, especially for nonlinear or multiphoton emissions, significantly improves the quality of the communication transmission while

10   minimizing self-focusing, self phase modulation and possible destruction of the fiber. The pulse characteristic control of ultrafast laser beam pulses, as described in all of the embodiments herein, should minimize, if not prevent, multiplicative noise effect disruption of nonlinear propagation channels in fiberoptic lines, as discussed in Mitra, et al., "Nonlinear Limits to the Information Capacity of Optical Fibre

15   Communications," *Nature*, vol. 411, pp. 1027-1030 (June 28, 2001). It is further envisioned that this type of pulse shaping apparatus can be employed within salt water oceans for submarine-to-submarine communications using shorter than 1 ps laser pulses.

[00102] While the preferred embodiment of the control system and

20   apparatus of the present invention have been disclosed, it should be appreciated that various modifications can be made without departing from the spirit of the present invention. For example, other laser beam pulse characteristics can be varied and

employed with the present invention beyond the pulse shaping and duration characteristics preferably described. Furthermore, additional software subroutines and statistical analyses can be employed. Moreover, other optical and pulse shaping devices can be used in place of those described, such as deformable mirrors and the

5   like. Finally, analog, solid state and fiber optic electrical control circuits can be substituted for or used in addition to a microprocessor and other computer circuitry. Various optics, including lenses and mirrors, can be used to achieve collimation or focusing. Additionally, dispersive optics, such as gratings and prisms, can be interchanged. Detection of the laser induced processes may use various

10   spectroscopic methods including laser induced fluorescence, Raman spectroscopy, nuclear magnetic resonance, gas chromatography, mass spectrometry and absorbtion spectroscopy. While various materials, specimens and components have been disclosed, it should be appreciated that various other materials, specimens and components can be employed. It is intended by the following claims to cover these

15   and any other departures from the disclosed embodiments which fall within the true spirit of this invention.